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Functional and structural analysis of catalase oxidized by singlet oxygen

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Abstract

Purified catalase-1 (CAT-1) from *Neurospora crassa* asexual spores is oxidized by singlet oxygen giving rise to active enzyme forms with different electrophoretic mobility. These enzyme forms are detected in vivo under stress conditions and during development at the start of the asexual morphogenetic transitions. CAT-1 heme *b* is oxidized to heme *d* by singlet oxygen. Here, we describe functional and structural comparisons of the non-oxidized enzyme with the fully oxidized one. Using a broad H_2O_2 concentration range (0.01–3.0 M), non-hyperbolic saturation kinetics was found in both enzymes, indicating that kinetic complexity does not arise from heme oxidation. The kinetics was consistent with the existence of two kinds of active sites differing more than 10-times in substrate affinity. Positive cooperativity for one or both of the saturation curves is possible. Kinetic constants obtained at 22 °C varied slightly and apparent activation energies for the reaction of both components are not significantly different. Protein fluorescence and circular dicroism of the two enzymes were nearly identical, indicating no gross conformational change with oxidation. Increased sensitivity to inhibition by cyanide indicated a local change at the active site in the oxidized catalase. Oxidized catalase was less resistant to high temperatures, high guanidinium ion concentration, and digestion with subtilisin. It was also less stable than the non-oxidized enzyme at an acid pH. The overall data show that the oxidized enzyme is structurally different from the non-oxidized one, although it conserves most of the remarkable stability and catalytic efficiency of the non-oxidized enzyme. Because the enzyme in the cell can be oxidized under physiological conditions, preservation of functional and structural properties of catalase could have been selected through evolution to assure an active enzyme under oxidative stress conditions.

Keywords: Two-component saturation kinetics; Cyanide sensitivity; Heme oxidation; Heme *d*; Singlet oxygen; Catalase stability; *Neurospora crassa*

1. Introduction

Many bacteria, fungi, and plants have more than one catalase (EC 1.11.1.6, hydrogen peroxide:hydrogen peroxide oxido-reductase). Catalases are differently regulated and some catalases are related to development [1–13]. Catalase activity during the *Neurospora crassa* asexual life cycle increases stepwise; asexual spores (conidia) have 60 times more catalase activity than hyphae growing in a liquid medium. This large increase in catalase activity is due to catalase-1 (CAT-1) synthesis during conidia formation and maturation [10]. The activity of another catalase, catalase-3, increases at the end of exponential growth and during adhesion of hyphae [10]. A null mutant strain of catalase-3 greatly increases aerial hyphae and conidia formation [12]. These results support the hypothesis that cell differentiation in *N. crassa* is a response to oxidative stress [14,15] and highlight the importance of catalases in cell differentiation.

Catalase is one of the most efficient enzymes known. Although extensively studied, many open questions remain to be answered for an enzyme that catalyzes an apparently very simple reaction: dismutation of two hydrogen peroxide (H_2O_2) molecules into two water and a dioxygen molecules. Small catalases (subunit of ≈60 kDa) are reversibly inhibited

Abbreviations: CAT-1, *Neurospora crassa* catalase-1; CAT-1a, the non-oxidized CAT-1; CAT-1e, the fully oxidized CAT-1; HPII, *Escherichia coli* hydroperoxidase II; PAGE, polyacrylamide gel electrophoresis; PB, phosphate buffer; SDS, sodium dodecyl sulfate; KCN, potassium cyanide.

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and irreversibly inactivated by substrate. In contrast, large catalases (subunit of ≈80 kDa) resist molar concentrations of H_2O_2 [16,17]. Resistance to substrate inhibition and inactivation in large catalases is in part due to a low rate formation of inactive compound II and III [18]. In contrast of not being inhibited by substrate, large catalase activity is actually stimulated by H_2O_2 at increasing concentrations [16–18]. The activation mechanism is not known.

CAT-1 is a homotetrameric, glycosylated, and large catalase. It is an enormously efficient enzyme, which is not inhibited by molar concentrations of H_2O_2. It also exhibits an unusual resistance to denaturation by temperature and various denaturants [16]. The enzyme is irreversibly oxidized in the course of few weeks of storage. This is accompanied by increased enzyme mobility in native polyacrylamide gel electrophoresis (PAGE), yet it preserves its molecular mass and its activity. In a zymogram of the gel, five activity bands can be observed depending on the degree of oxidation. The less acidic band corresponds to the non-oxidized enzyme (CAT-1a) (p*I* 5.45), the more acidic band corresponds to the fully oxidized (CAT-1e) (p*I* 5.25); CAT-1b to CAT-1d are intermediate forms [19]. Storage under argon prevents CAT-1 oxidation. Exposure of purified CAT-1a to a pure source of singlet oxygen causes a rapid oxidation and the characteristic change in electrophoretic mobility; no other reactive oxygen species could generate a similar electrophoretic shift [19].

Catalases assayed from bacterial, fungal, plant, or animal sources are likewise oxidized by singlet oxygen producing increase in enzyme electrophoretic mobility [19]. In *N. crassa*, the large catalase-3 [10] and a catalase-peroxidase, catalase-2 [19], which is a completely different heme–enzyme, are similarly oxidized by singlet oxygen, causing a shift in their electrophoretic mobility. However, the electrophoretic shift in these enzymes is much smaller than in CAT-1.

Oxidation of CAT-1 occurs in vivo under conditions in which singlet oxygen is generated [20]. Human catalase oxidation by singlet oxygen takes place in cultures of myeloid cell (U937) [21]. In fact, because it is exclusively generated by singlet oxygen, the change in electrophoretic mobility of catalases can be used to detect singlet oxygen formation inside cells [22].

A site of modification in CAT-1 is heme: UV-visible absorbance spectra of the heme indicated that singlet oxygen modification of the enzyme increased heme asymmetry [19], which is consistent with heme *b* to heme *d* transformation. The crystal structure of CAT-1 indicates modification of heme *b* to a *cis*-hydroxyl-γ-spirolactone at the porphyrin ring III (heme *d*) [23] like in the two other large catalases, *Escherichia coli* HPII and *Penicillium vitale,* catalase, whose tri-dimensional structure is known [24]. Enzyme activity is required for heme *b* to heme *d* oxidation [25]. Our hypothesis is that singlet oxygen at the active site reacts with heme *b* to form heme *d*.

Because oxidation of heme per se does not change protein charge, modification of one or few charged amino acid residues, or loss of a charged protein end, could explain the electrophoretic shift. No modification of amino acids was observed in the crystal structure of CAT-1 [23], however the N-terminal loose end in the structure is not visible and a modification at a residue situated in this end might have taken place. Another possibility is that heme modification causes a structural change that modifies the charge of the protein.

To investigate the functional and structural consequences of catalase oxidation, we compared the kinetics, some structural properties, and the stability of the non-oxidized enzyme (CAT-1a) with those of the fully oxidized one (CAT-1e). Both enzymes exhibited complex saturation kinetics by H_2O_2 with similar kinetic constants and activation energies. Slight structural differences between CAT-1e and CAT-1a and differences in stability between them were detected under extreme situations. However, CAT-1e conserves most of the remarkable stability and catalytic efficiency of the non-oxidized enzyme. Because the enzyme in the cell can be oxidized under physiological conditions, preservation of functional and structural properties of catalase could have been selected through evolution to assure an active enzyme under oxidative stress conditions.

2. Materials and methods

2.1. Chemicals, strains, and culture conditions

Chemicals were from Sigma unless otherwise stated.

N. crassa, wild type strain 74-OR23-1A from the Fungal Genetic Stock Center, was grown from stocks of conidia kept in distilled water at –70 °C. Conidia were inoculated on agar minimal-medium of Vogel supplemented with 1.5% sucrose. Cultures were grown for 3 days in the dark at 30 °C followed by 2 days in the light at 25 °C.

2.2. Purification of CAT-1

CAT-1 was purified from conidiated aerial mycelium as described previously [16,19]. Briefly, cultures were harvested directly in acetone and collected by filtering through filter paper. Dried acetone powders were ground with glass beads in a cold buffer containing protease inhibitors and antioxidants. Cell extract was frozen and thawed twice and, after centrifugation, the supernatant was precipitated with cold acetone. The resuspended pellet was centrifuged and the supernatant was precipitated with 35% (w/v) ammonium sulfate. The dissolved pellet was passed through a Phenyl Sepharose CL-4B (Pharmacia) column and eluted with 10 mM phosphate buffer (PB), pH 7.8, adjusted by mixing Na_2HPO_4 and KH_2PO_4 solutions. The purified enzyme gave a yield of 48% of the total cell extract activity and an increase in catalase specific activity of 170 times. After PAGE with sodium dodecyl sulfate (SDS) and staining the gel with Coomassie Brilliant Blue, the preparation showed a main band of 88 kDa and few minor bands [16].